AD_____

Award Number DAMD17-98-1-8037

TITLE: The Target Sites on EGF Receptor for CBL , Its Negative Regulator

PRINCIPAL INVESTIGATOR: Christopher E. Andoniou, Ph.D.

CONTRACTING ORGANIZATION: Brigham and Women's Hospital
Boston, Massachusetts 02115

REPORT DATE: April 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 1999	Annual Summary (1 Apr 98 - 31 Mar 99)

4. TITLE AND SUBTITLE
The Target Sites on EGF Receptor for Cbl, Its Negative Regulator

5. FUNDING NUMBERS
DAMD17-98-1-8037

6. AUTHOR(S)
Christopher E. Andoniou, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brigham and Women's Hospital
Boston, Massachusetts 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Recent studies have provided evidence that the Cbl oncogene acts as a negative regulator of the EGFR, by enhancing the rate of degradation of the activated receptor. I have determined that Cbl is a potent negative regulator of the Src family kinase Fyn. Cbl's effect is mediated, at least in part be enhancing the rate of Fyn protein turnover. A known transforming mutant of cbl, 70Z-Cbl is incapable of performing this function. Interestingly, Src family kinases are known to be activated by ErbB family members and to enhance the mitogenic response mediated by this family of kinases. Paradoxically, Src has been shown to enhance the rate of internalization of the activated EGFR, but does not alter its rate of degradation. Thus, Src kinases may be involved in regulating the intracellular trafficking or the subcellular localization of activated EGFR. Cbl-mediated negative regulation of Src kinases would provide a novel mechanism by which Cbl could regulate the activated EGFR, and potentially other ErbB family members.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Andonian 30-Apr 99

PI - Signature Date

Table of Contents:

The Target Sites on the EGF Receptor for Cbl, its negative regulator

The experiments described in the application for funding were designed to test the hypothesis that the proto-oncogene product Cbl functions as a negative regulator of the activated epidermal growth factor receptor (EGFR). Genetic studies in C. elegans and Drosophila identified the Cbl homologues SLI-1 and D-Cbl as being negative regulators of EGFR-mediated developmental events in these organisms. Observations in mammalian systems identified Cbl as a substrate for the activated EGFR and showed that Cbl is rapidly recruited to the activated EGFR. We have shown the N-terminal portion of Cbl encodes a novel phosphotyrosine-binding motif (termed a tyrosine kinase-binding domain, TKB), which is capable of directly binding a number of activated tyrosine kinases including the EGFR. More recently this portion of Cbl was crystallized and shown to encode a novel SH2 like domain (Meng et al., 1999). Taken together these results implicated Cbl as a candidate for mediating negative regulation of the EGFR.

This hypothesis was to be tested by defining the site(s) within the EGFR responsible for mediating binding to the Cbl TKB domain. These mutant receptors were then to be expressed in $EGFR^-$ fibroblasts to determine if abrogation of the Cbl binding site would result in a hyper-responsive receptor. Screening of a degenerate phosphopeptide library with the Cbl TKB domain identified the sequence D(D/N)XpY as a preferred binding motif (Lupher et al., 1997). A number tyrosines in the EGFR conform to this sequence motif specifically Y992, a known autophosphorylation site and Y974, Y1068 and Y1086. Thus, mutant EGFR's were constructed in which these tyrosines were mutated to phenylanine and the resultant constructs overexpressed in 293T cells. Cells were then either stimulated with EGF or left unstimulated and total cellular extracts prepared. These were then used for *in vitro* binding assays to determine if they were still capable of being recognized by the Cbl TKB domain. The result of these experiments were that no mutant tested abrogated the Cbl TKB domain interaction. The mutant receptors tested were Y974F, Y992F, Y1068F, Y1086F and the combination mutants Y974F/Y992F, Y992F/1086F, Y976F/1086F and Y1068F/1086F.

However, two recent publications required a substantial change in the direction of the project. In my mentor's laboratory, Miyake et al. (1998) showed that Cbl functions as a negative regulator by enhancing the rate of degradation of the PDGFRα, a receptor tyrosine kinase related to EGFR. More directly, Levkowitz and colleagues and Dr N. Lill in my mentor's laboratory demonstrated that Cbl also functions as negative regulator of the EGFR by enhancing its rate of degradation. These data, indicated that Cbl did not increase the rate at which the EGFR was internalized, but rather increased the proportion of EGFR targeted to endosomes and lysosomes.

Furthermore, oncogenic forms of Cbl inhibited down-regulation of the EGFR by promoting the recycling of the internalized receptor back to the cell surface. Taken together these results suggested that Cbl may be acting once the receptor has been internalized and possibly at the sorting endosome stage.

Given these results, I have begun to investigate the possibility that Cbl functions as a negative regulator of the Src family of kinases, and as such can influence the intracellular trafficking of internalized EGFR. The Src family members c-Src, Fyn and c-Yes are widely expressed and are activated by diverse cellular stimuli. Significantly, the tyrosine kinase activity of all three family members is increased by EGF stimulation and Src has been shown to associate with the EGFR and ErbB-2 (Neu) (Osherov and Levitzki, 1994; Muthuswamy et al., 1994). Even more importantly, levels of Src family members or their activity have been found to be significantly increased in a substantial proportion of cases of breast and colon cancer. Similarly, an increase in Src kinase activity was detected in Neu-induced mammary tumors in a transgenic mouse model and overexpression of Src in fibroblasts has been shown to enhance EGF induced mitogenesis and tumor formation in nude mice (Muthuswamy and Muller, 1995; Maa et al., 1995). The striking correlation between enhanced ErbB activity and elevated Src kinase activity has implicated the Src family of proteins as having a possible role in the generation or progression of human tumors such as breast cancer.

Surprisingly, overexpression of Src was also found to enhance the endocytic internalization of the EGFR but did not alter the half-life of the protein (Ware et al., 1997). Thus an apparent contradiction exists; Src enhances mitogenic responses initiated by EGFR, but also increases the rate at which the receptor is internalized, which is thought to be a method of signal termination. A clue to understating this phenomenon may come from the observation that both Src and Fyn are known to localize to the microtubual organizing center, a site known to be important in the regulation of recycling of proteins back to the cell surface. Thus a possible explanation for these results is that Src kinases enhance the mitogenic response by increasing the rate of recycling of internalized receptors or by altering the intracellular localization of the activated receptors and thereby prolonging the mitogenic signal.

Given that Cbl is known to associate with Src kinases, an intriguing possibility is that Cbl may negatively regulate the EGFR indirectly by affecting the kinase activity of Src kinases and thereby increasing the proportion of EGFR targeted for lysosomal degradation. This possibility was initially tested using the Fyn tyrosine kinase in transient transfection assays. I have determined that overexpression of Cbl in 293T cells is capable of reducing the proportion of

kinase-active Fyn. Cbl is able to downregulate both wild-type Fyn and a constitutively active mutant of Fyn in which the negative regulatory tyrosine has been mutated to phenylalanine (FynY528F). These results were obtained by transient overexpression in 293T cells in which two concentrations of Fyn or FynY528F expression plasmid were transfected, either alone or in combination with a Cbl expression construct. A CD8-ζ chimera was also transfected to act as an endogenous substrate. This experiment demonstrated that expression of Fyn or FynY528F in these cells resulted in the efficient phosphorylation of CD8-ζ and the phosphorylation of an endogenous substrate of 62kD (p62) (Fig 1). Expression of Cbl resulted in a dramatic reduction in the tyrosine phosphorylation of Fyn, CD8-ζ and endogenous p62. Surprisingly, overexpression of Cbl in combination with Fyn seemed to cause a reduction in Fyn protein levels; this is particularly evident when FynY528F was used (Fig 1, middle panel). I then went on to test 70Z-Cbl, a transforming version of Cbl, in this assay and found that, unlike wild-type Cbl, 70Z-Cbl did not reduce the tyrosine phosphorylation content of Fyn, CD8-ζ or p62; in fact a slight increase in these signals was observed (Fig 2). Furthermore overexpression of 70Z-Cbl did not reduce Fyn protein levels even though this protein is capable of associating with Fyn (data not shown). Finally, overexpression of Cbl has no effect on the protein levels of kinase-dead Fyn in this assay, even though Cbl is capable of associating with Fyn as measured by coimmunoprecipitation studies (data not shown).

Taken together these results indicated that Cbl is capable of negatively regulating the Fyn tyrosine kinase and suggested that the method of this regulation was by enhancing the rate of Fyn protein turnover. This was tested by a pulse-chase experiment in which FynY528F was expressed in 293T cells, either alone or in combination with Cbl. Cells were pulse labeled with ^{35}S-methionine for 30 minutes, and the labeled cells were then grown in normal medium (supplemented with excess non-radioactive methionine) for the indicated chase times. Fyn protein from these cells was isolated by immunoprecipitation, separated by SDS-PAGE and transferred to PVDF membrane. Fyn protein was visualized by exposing the membrane to X-ray film and the signals quantified by PhosphorImager (Fig 3). This experiment clearly demonstrated that Cbl expression reduced the half-life of Fyn ($T_{1/2}$=570 min without Cbl; $T_{1/2}$=140 with Cbl).

These results clearly demonstrate that Cbl is capable of negatively regulating the Fyn tyrosine kinase, and that this effect is mediated, at least in part by Cbl enhancing the rate at which activated Fyn is degraded. The question then arises: is the increase in protein turnover the result of Cbl directly promoting degradation or an indirect effect caused by Cbl downregulating

the kinase activity. Initial experiments using *in vitro* kinase assays suggest that Cbl has no effect on the kinase activity of Fyn and that Cbl regulates Fyn primarily by increasing Fyn degradation. A number of previous reports have identified Cbl as being a substrate for and interacting with Src and so I intend to extend this study be deterring if Cbl is capable of also regulating Src.

The modified longer terms goal of this project are to determine if:

- Src or Fyn overexpression alters the proportion of activated EGFR targeted for lysosomal degradation and/or its subcellular distribution
- Determine if Cbl inhibition of Src kinases alters the subcelluar localization of the EGFR.
- To characterize the compartment in which activated EGFR is localized as a result of its interaction with Src family kinases as this represents a novel intracellular location where crucial positive signals are transmitted by ErbB receptors.

These possibilities will be initially tested by transient overexpression of Src or Fyn in Cos cells and determining if these proteins alter the trafficking or localization of endogenous EGFR. This will be performed by subcellular fractionation of cells followed by western blotting of these fractions. Confocal microscopy will be used to complement the fractionation studies.

Cbl and its mutants will then be used in this transfection system to determine if Cbl negative regulation of Src or Fyn influences the trafficking or localization of EGFR.

This line of investigation is particularly exciting since receptor internalization is currently thought to result in the termination of signaling from receptor tyrosine kinases. This study may modify the current paradigm of receptor tyrosine kinase signaling and allow the design of novel therapeutic agents, which influence the rate at which EGFR and other ErbB family members are internalized and degraded.

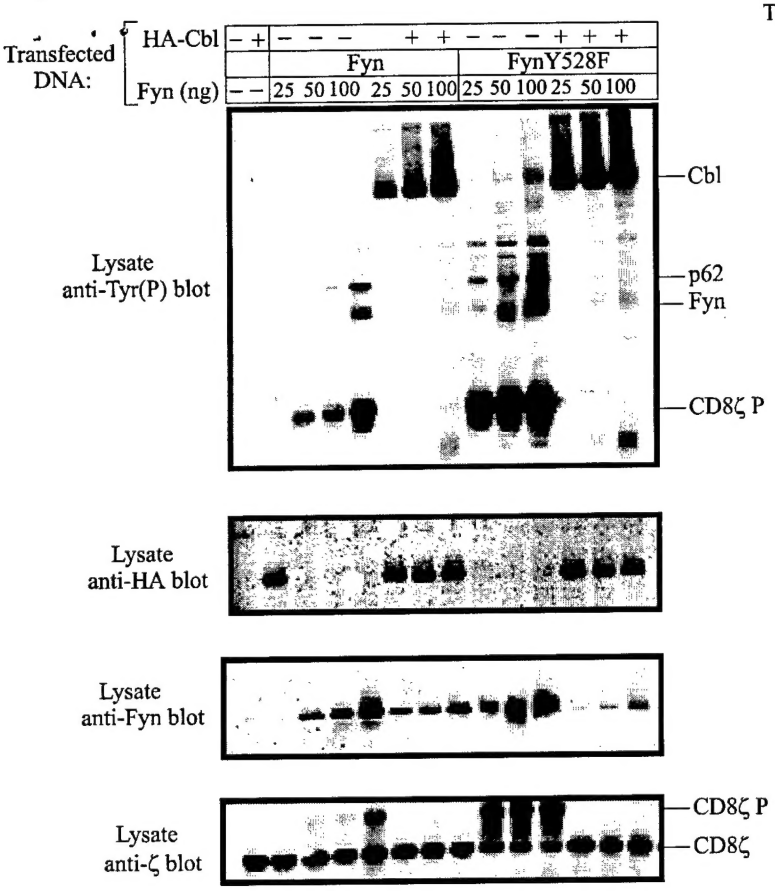


Figure 1. Overexpression of Cbl reduces the activity and amount of coexpressed Fyn or FynY528F.
293T cells were transfected with pSRαneo-CD8-ζ (0.5μg) and the indicated amounts of pAlterMAX constructs encoding Fyn or FynY528F, with or without pAlterMAX-HA-Cbl (1μg). At 48 h after transfection, cells were lysed and 15μg aliquots of cell lysate were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted with the indicated antibodies.

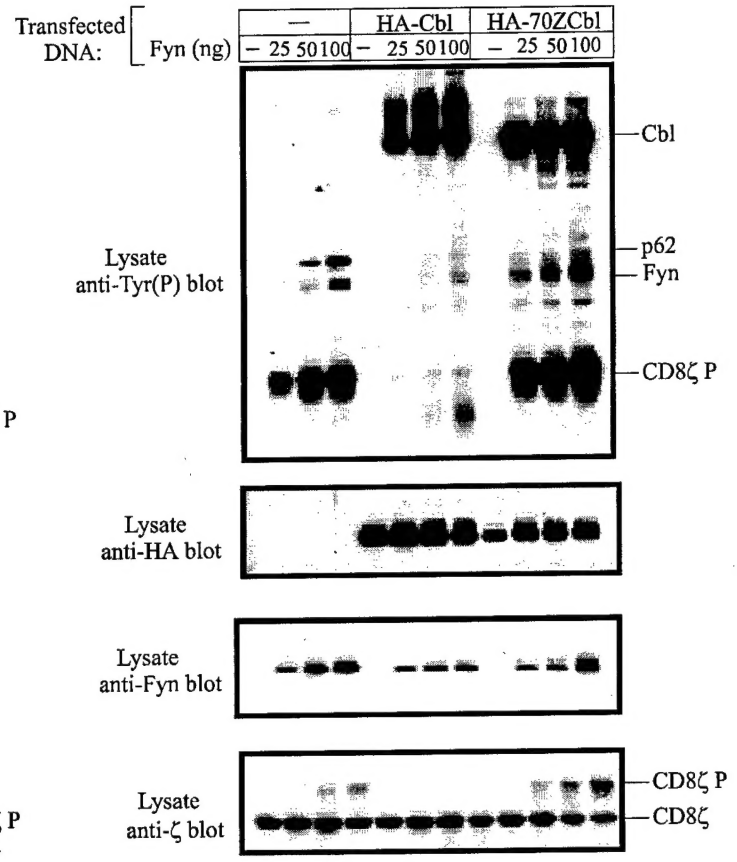
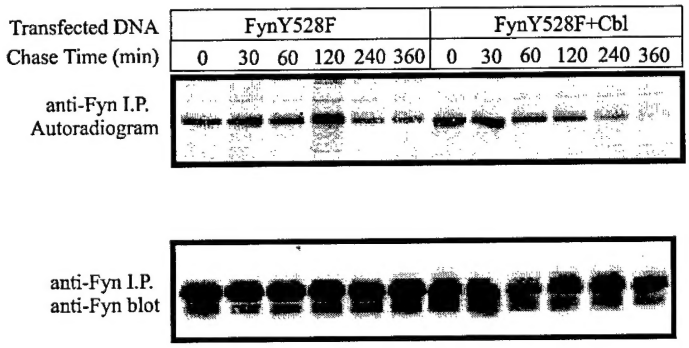


Figure 2. The transforming protein 70Z-Cbl does not reduce Fyn protein levels or affect phosphorylation of *in vivo* Fyn substrates.
293T cells in 100 mm tissue culture dishes were transfected with pSRαneo-CD8-ζ (0.5μg), the indicated amounts of pAlterMAX-Fyn, either alone, or in combination with 1μg of pAlterMAX-HA-Cbl or pAlterMAX-HA-70Z-Cbl expression construct. Cell lysates were prepared 48 h after transfection, and 15μg aliquots were resolved by SDS-PAGE. The polypeptides were transferred to PVDF membrane and immunoblotted with the indicated antibodies.



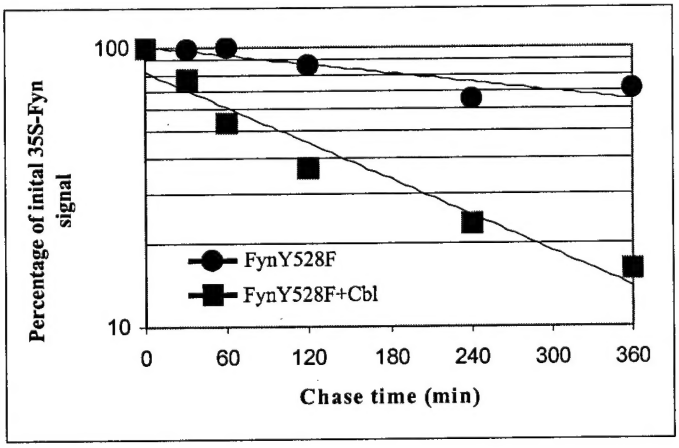


Figure 3. Cbl overexpression enhances the turnover of FynY528F protein as determined by metabolic pulse-chase analysis.
293T cells in 100 mm tissue culture dishes were transfected with pAlterMAX-FynY528F (0.1 μg) with or without the pAlterMAX-HA-Cbl expression construct (1 μg). At 48 h post-transfection, cells were methionine-starved for 1h and then were pulse-labeled with ^{35}S-methionine for 45 min. Pulse-labeled cells were chased in culture medium supplemented with methionine for the indicated times, and cell lysates were prepared. Anti-Fyn immunoprecipitates from 300μg aliquots of cell lysates were resolved by SDS-PAGE and transferred to PVDF membrane. Labeled Fyn protein was visualized by exposing the membrane to X-ray film for 24 h (top left). The membrane was then immunoblotted with an anti-Fyn antibody (left bottom). The membrane shown left was used for quantification of ^{35}S-labeled Fyn on an FLA-2000 PhosphorImager. The values at various chase times are expressed as a percentage relative to ^{35}S-Fyn signals at time 0. The line of best fit was calculated using Microsoft Excel.